Remarks

Attorney Gail M. Kempler and Examiner Eileen B. O'Hara held a Telephone Interview on June 8, 2001, in connection with USSN 09/313,942. During the Interview, it was decided that the Applicants would file a Continued Prosecution Application for 09/313,942 and submit a Terminal Disclaimer in connection with the CPA. For the Examiner's convenience, a copy of the Telephone Interview Summary dated June 8, 2001, is provided herewith as Exhibit B. Also provided, as Exhibit C, is the required Terminal Disclaimer and Statement Under 37 CFR § 3.73(b). Applicants contend that, with the filing of a CPA and a Terminal Disclaimer, Applicants have overcome the rejection under 35 USC §103(a) that was issued in the Office Action mailed on February 27, 2001 and the double-patenting rejection that was issued in the Office Action mailed on August 14, 2000, both in connection with USSN 09/313,942. Therefore, Applicants respectfully request that the Examiner withdraw the rejections and issue a Notice of Allowance for all of the pending claims.

No fee, other than the $110.00 fee for a filing a Terminal Disclaimer, is deemed necessary in connection with filing this Preliminary Amendment and Response. However, if any additional fee is necessary, authorization is hereby given to charge the amount of any such additional fee to Deposit Account No. 18-0650.

Respectfully submitted,

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MARKED-UP VERSION

RECEPTOR BASED ANTAGONISTS AND
METHODS OF MAKING AND USING

This application claims priority of U.S. Provisional application No. 60/101,858 filed September 25, 1998. Throughout this application various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application.

BACKGROUND OF THE INVENTION

Although discovered for varying biological activities, ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), oncostatin M (OSM) and interleukin-6 (IL-6) comprise a defined family of cytokines (referred to herein as the "CNTF family" of cytokines). These cytokines are grouped together because of their distant structural similarities [Bazan, J. Neuron 7: 197-208 (1991); Rose and Bruce, Proc. Natl. Acad. Sci. USA 88: 8641-8645 (1991)], and, perhaps more importantly, because they share "β" signal-transducing receptor components [Baumann, et al., J. Biol. Chem. 265:19853-19862 (1993); Davis, et al., Science 260: 1805-1808 (1993); Gearing et al., Science 255:1434-1437 (1992); Ip et al., Cell 69: 1121-1132 (1992); Stahl, et al., J. Biol. Chem. 268: 7628-7631 (1993); Stahl and Yancopoulos, Cell 74: 587-590 (1993)]. Receptor activation by this family of cytokines results from either homo- or hetero-dimerization of these β components [Davis, et al. Science 260: 1805-1808 (1993), Murakami, et al., Science 260: 1808-1810 (1993); Stahl and Yancopoulos, Cell 74: 587-590 (1993)]. IL-6 receptor activation requires homodimerization of gp130 [Murakami, et al. Science 260: 1808-1810 (1993), Hibi, et al., Cell 63: 1149-1157 (1990)], a protein initially identified as the IL-6 signal transducer [Hibi, et al., Cell 63: 1149-1157

induced activation of the IL-6 receptor system, which is blocked upon coaddition of CNTF.

FIGURE 3: Scatchard analysis of iodinated CNTF binding on PC12D cells. PC12D cells were incubated with various concentrations of iodinated CNTF in the presence or absence of excess non-radioactive competitor to determine the specific binding. The figure shows a Scatchard plot of the amount of iodinated CNTF specifically bound, and gives data consistent with two binding sites with dissociation constants of 9 pM and 3.4 nM.

A-B (SEQ ID NO:7) (SEQ ID NO:7)

FIGURE 4: The amino acid sequence of human gp130-Fc-His_6. Amino acids 1 to 619 are from human gp130 (Hibi et al., Cell 63:1149-1157 (1990). Note that amino acid number 2 has been changed from a Leu to a Val in order to accommodate a Kozak sequence in the coding DNA sequence. The signal peptide of gp130-Fc-His_6 has been italicized (amino acids 1 to 22). The Ser-Gly bridge is shown in bold type (amino acids 620, 621). Amino acids 662 to 853 are from the Fc domain of human IgG1 (Lewis, et al., J. Immunol. 151:2829-2838 (1993). (†) mark the two cysteines (amino acids number 632 and 635) of the IgG hinge preceding the Fc that form the inter-chain disulfide bridges that link two Fc domains. The hexahistine tag is shown in bold/italic type (amino acids 854 to 859). (•) shows the position of the STOP codon.

(SEQ ID NO:8) (SEQ ID NO:8)

FIGURE 5: The amino acid sequence of human IL-6Rα-Fc. Key: Amino acids 1 to 358 are from human IL-6Rα (Yamasaki, et al., Science 241:825-828 (1988). Note that amino acid number 2 has been changed from a Leu to a Val in order to accommodate a Kozak sequence in the coding DNA sequence. The signal peptide of IL-6Rα-Fc has been italicized (amino acids 1 to 19). The Ala-

Gly bridge is shown in bold type (amino acids 359, 360). Amino acids 361 to 592 are from the Fc domain of human IgG1 (Lewis et al., J. Immunol. 151:2829-2838 (1993). (†) mark the two cysteines (amino acids number 371 and 374) of the IgG hinge preceding the Fc that form the inter-chain disulfide bridges that link two Fc domains. (•) shows the position of the STOP codon.

FIGURE 6: The CNTF/IL-6/IL-11 receptor system. The ordered formation of the hexameric signal transducing receptor complex is depicted schematically. The cytokine associates with the Rα component to form an obligatory cytokine•Rα complex (Kd is about 5 nM). This low affinity complex next associates with the first signal transducing component, marked β1, to form a high affinity cytokine•Rα•β1 complex (Kd is about 10 pM). In the case of IL-6Rα, this component is gp130. This trimeric high affinity complex subsequently associates with another such complex. Formation of this complex results in signal transduction as it involves dimerization of two signal transducing components, marked β1 and β2 respectively (adapted from (Ward et al., J. Bio. Chem. 269:23286-23289 (1994); Stahl and Yancopoulos, J. Neurobiology 25:1454-1466 (1994); Stahl and Yancopoulos, Cell 74:587-590 (1993).

FIGURE 7: Design of heterodimeric receptor-based ligand traps for IL-6. The heterodimeric ligand trap is comprised of two interdisulfide linked proteins, gp130-Fc and IL-6Rα-Fc. The gp130-Fc•IL-6Rα-Fc complex (upper panel) is shown to mimic the high affinity cytokine•Rα•β1 complex (lower panel). The ligand trap functions as an antagonist by sequestering IL-6 and thus rendering unavailable to interact with the native receptors on IL-6-responsive cells.

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FIGURE 8. Heteromeric immunoglobulin Heavy/Light Chain Receptor Fusions. An example of a heavy/light chain receptor fusion molecule is schematically depicted. The extracellular domain of gp130 is fused to Cγ, whereas the extracellular domain of IL-6Rα is fused to the constant region of the kappa chain (κ). The inter-chain disulfide bridges are also depicted (S-S).

A-B (SEQ ID NO:9) (SEQ ID NO:9)

FIGURE 9. Amino acid sequence of gp130-Cγ1. Key: Amino acids 1 to 619 are from human gp130 (Hibi, et al., Cell 63:1149-1157 (1990). Ser-Gly bridge is shown in bold type. Amino acids 662 to 651 are from the constant region of human IgG1 (Lewis et al., J. Immunol. 151:2829-2838 (1993). (*) shows the position of the STOP codon.

(SEQ ID NO:10) (SEQ ID NO:10)

FIGURE 10. Amino acid sequence of gp130Δ3fibro. Key: Amino acids 1 to 330 are from human gp130 (Hibi et al., Cell 63:1149-1157 (1990). Other symbols as described in Figure 9. A-B (SEQ ID NO:9)

(SEQ ID NO:11) (SEQ ID NO:11)

FIGURE 11. Amino acid sequence of J-CH1. Key: The Ser-Gly bridge is shown in bold, the J-peptide is shown in italics, the C_H1 domain is underlined.

(SEQ ID NO:12) (SEQ ID NO:12)

FIGURE 12. Amino acid sequence of Cγ4. Key: The Ser-Gly bridge is shown in bold type. Amino acids 2 to 239 comprise the Cγ4 sequence.

(SEQ ID NO:13) (SEQ ID NO:13)

FIGURE 13. Amino acid sequence of κ-domain. Key: The Ser-Gly bridge is shown in bold type. Amino acids 2 to 108 comprise the κ domain. The C-terminal cysteine (amino acid 108) is that involved in the disulfide bond of the κ domain with the C_H1 domain of Cγ.

FIGURE 14. Amino acid sequence of λ-domain. (SEQ ID NO: 14) Key: The Ser-Gly bridge is shown in bold type. (SEQ ID NO: 14) Amino acids 2 to 106 comprise the λ domain (Cheung, et al., J. Virol. 66: 6714-6720 (1992). The C-terminal cysteine (amino acid 106) is that involved in the disulfide bond of the λ domain with the C_H1 domain of Cγ.

FIGURE 15. Amino acid sequence of the soluble IL-6Rα domain. (SEQ ID NO:15) Key: Amino acids 1 to 358 comprise the soluble IL-6Rα domain (Yamasaki, et al., Science 241:825-828 (1988). The Ala-Gly bridge is shown in bold type. (SEQ ID NO:15)

FIGURE 16. Amino acid sequence of the soluble IL-6Rα313 domain: Key: Amino acids 1 to 313 comprise the truncated IL-6Rα domain (IL-6Rα313). The Thr-Gly bridge is shown in bold type. (SEQ ID NO:16) (SEQ ID NO:16)

FIGURE 17: Purification of gp130-Cγ1•IL-6Rα-κ. 4% to 12% SDS-PAGE gradient gel run under non-reducing conditions. Proteins were visualized by staining with silver. Lane 1: approximately 100 ng of material purified over Protein A Sepharose (Pharmacia). Lane 2: Molecular size standards (Amersham). Lane 3: The Protein A-purified material shown here after further purification over an IL-6 affinity chromatography step. The positions of the gp130-Cγ1 dimer [$(gp130\text{-}C\gamma1)_2$], the gp130-Cγ1 dimer associated with one IL-6Rα-κ [$(gp130\text{-}C\gamma1)_2\bullet(IL\text{-}6R\alpha\text{-}\kappa)_1$], and the gp130-Cγ1 dimer associated with two IL-6Rα-κ [$(gp130\text{-}C\gamma1)_2\bullet(IL\text{-}6R\alpha\text{-}\kappa)_2$] are shown, as well as the sizes for the molecular size standards in kilodaltons (200, 100, and 46).

FIGURE 18. IL-6 dissociates slowly from the ligand trap. The dissociation

rate of IL-6 from a heavy/light chain receptor-based ligand trap (gp130-Cγ1•IL-6Rα-κ) was compared to that obtained with the neutralizing monoclonal antibody B-E8 (BE8 MAb).

FIGURE 19 A-B. IL-6 can induce multimerization of the ligand trap. (A) Figure 19 Two different ligand traps are depicted schematically and listed according to their ability to bind protein A. gp130-Fc•IL-6Rα-Fc (GF6F) binds protein A via its Fc-domains, whereas gp130-C_H1•IL-6Rα-κ (G16K) does not bind to protein A. (B) Figure 19 Anti-kappa western blotting of proteins precipitated with Protein A-Sepharose from mixtures of GF6F ± IL-6, G16K ± IL-6, or GF6F plus G16K ± IL-6, as marked.

FIGURE 20. Inhibition of IL-6-dependent XG-1 cell proliferation. XG-1 cells [Zhang, et al., Blood 83:3654-3663 (1994)] were prepared for a proliferation assay by starving the cells from IL-6 for 5 hours. Assays were set up in 96-well tissue culture dishes in RPMI + 10% fetal calf serum + penicillin/streptomycin + 0.050 nM 2-mercaptoethanol + glutamine. 0.1 ml of that media was used per well. Cells were suspended at a density of 250,000 per ml at the start of the assay. 72 hours post addition of IL-6 ± ligands traps or antibodies, an MTT assay was performed as described (Panayotatos et al. Biochemistry 33:5813-5818 (1994). The different ligand traps utilized are listed.

FIGURES 21A-21D (SEQ ID NOS: 17 and 18) - Nucleotide sequence (SEQ ID NO: 17) encoding and deduced amino acid sequence (SEQ ID NO: 18) of fusion polypeptide designated 424 which is capable of binding the cytokine IL-4 to form a nonfunctional complex.

FIGURES 22A-22D (SEQ ID NOS: 19 and 20) - Nucleotide sequence (SEQ ID NO: 19) encoding and deduced amino acid

(SEQ ID NO:20)
sequence of fusion polypeptide designated 603 which is capable of binding the cytokine IL-4 to form a nonfunctional complex.

(SEQ ID NOS: 21 and 22) (SEQ ID NO:21)
FIGURES 23A-23D - Nucleotide sequence encoding and deduced amino acid
(SEQ ID NO: 22)
sequence of fusion polypeptide designated 622 which is capable of binding the cytokine IL-4 to form a nonfunctional complex.

(SEQ ID NOS: 23 and 24) (SEQ ID NO:23)
FIGURE 24A-24F - Nucleotide sequence encoding and deduced amino acid
(SEQ ID NO:24)
sequence of fusion polypeptide designated 412 which is capable of binding the cytokine IL-6 to form a nonfunctional complex.

(SEQ ID NOS: 25 and 26) (SEQ ID NO:25)
FIGURE 25A-25F - Nucleotide sequence encoding and deduced amino acid
(SEQ ID NO:26)
sequence of fusion polypeptide designated 616 which is capable of binding the cytokine IL-6 to form a nonfunctional complex.

(SEQ ID NOS: 27 and 28) (SEQ ID NO: 27)
FIGURE 26A-26E - Nucleotide sequence encoding and deduced amino acid
(SEQ ID NO: 28)
sequence of fusion polypeptide designated 569 which is capable of binding the cytokine IL-1 to form a nonfunctional complex.

FIGURE 27 - shows that an IL-4 trap designated 4SC375, which is a fusion polypeptide of IL-2Rγ-scb-IL4Rα-FcΔC1, is several orders of magnitude better as an IL-4 antagonist than IL4RαFcΔC1 alone in the TF1 cell bioassay.

FIGURE 28 - shows that an IL-4 trap designated 4SC375 displays antagonistic activity in the TF1 cell bioassay equivalent to an IL-4 trap designated 4SC424 (described in Figs. 21A-21D) which is a fusion polypeptide of IL-2Rγ-IL4Rα-FcΔC1 having the IL-2Rγ component flush with the IL-4Rα component.

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FIGURE 29 - shows that the IL6 trap (6SC412 IL6R-scb-gpx-FcΔC1) described in Figs. 24A-24F is a better antagonist of IL-6 in the XG1 bioassay than the neutralizing monoclonal antibody to human IL-6 - BE8.

FIGURE 30 - shows that the trap 1SC569 (described in Figs. 26A-26E) is able to antagonize the effects of IL-1 and block the IL-6 production from MRC 5 cells upon treatment with IL-1.

FIGURE 31A-31G (SEQ ID NOS: 29 and 30) (SEQ ID NO: 29) (SEQ ID NO: 30) - The nucleotide and encoded amino acid sequence of the IL-4Rα.IL-13Rα1.Fc single chain trap construct is set forth.

FIGURE 32A-32G (SEQ ID NOS: 31 and 32) (SEQ ID NO: 31) (SEQ ID NO: 32) - The nucleotide and encoded amino acid sequence of the IL-13Rα1.IL-4Rα.Fc single chain trap construct is set forth.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides an isolated nucleic acid molecule encoding a fusion polypeptide capable of binding a cytokine to form a nonfunctional complex comprising:

a) a nucleotide sequence encoding a first fusion polypeptide component comprising the amino acid sequence of the cytokine binding portion of the extracellular domain of the specificity determining component of the cytokine's receptor;

b) a nucleotide sequence encoding a second fusion polypeptide component comprising the amino acid sequence of the cytokine binding portion of the extracellular domain of the signal transducing component of the cytokine's receptor; and

c) a nucleotide sequence encoding a third fusion polypeptide component comprising the amino acid sequence of a multimerizing component.

washes with assay buffer. Cells were lysed with PBS containing 1% SDS and counted in a Packard Gamma Counter at 90-95% efficiency. Non-specific binding was defined by the presence of 100-fold excess of unlabelled CNTF. Specific binding ranged from 70% to 95%.

RESULTS

The equilibrium constant for binding of CNTF to CNTFRα:β1 was estimated from Scatchard analysis of iodinated CNTF binding on PC12D cells (Figure 3). The data is consistent with a 2 site fit having dissociation constants of 9 pM and 3.4 nM. The low affinity site corresponds to interaction of CNTF with CNTFRα , which has a Kd near 3 nM [(Panayotatos, et al., J. Biol. Chem. 268: 19000-19003 (1993)]. We interpret the high affinity complex as the intermediate containing CNTF, CNTFRα , and gp130. A Ewing sarcoma cell line (EW-1) which does contain CNTFRα, gp130, and LIFRβ, and therefore gives robust tyrosine phosphorylation in response to CNTF, displays a very similar two site fit with dissociation constants of 1 nM and 10 pM (Wong, et al., unpublished data). Thus it is apparent that CNTF binds with equally high affinity to a complex containing only CNTFRα and gp130, as it does to a complex which additionally contains LIFRβ, thus demonstrating the feasibility of creating the sRα:β antagonists described herein.

EXAMPLE 3. METHODS OF PRODUCING CYTOKINE LIGAND TRAPS

Virus Stock Production

SF21 insect cells obtained from *Spodoptera frugiperda* were grown at 27C in Gibco SF900 II medium to a density of $1x10^6$ cells/mL. The individual virus stock for either GP130-Fc-His_6 (Figures 4A and 4B [SEQ ID NO:7]) or IL6Ra-Fc (Figure 5 [SEQ ID NO:8]) was added to the bioreactor to a low multiplicity 0.01-0.1 PFU/cell to begin the

infection. The infection process was allowed to continue for 5-7 days allowing maximum virus replication without incurring substantial cell lysis. The cell suspension was aseptically aliquoted into sterile centrifuge bottles and the cells removed by centrifugation. The cell-free supernatant was collected in sterile bottles and stored at 4C until further use.

The virus titer was determined by plaque assay as described by O'Reilly, Miller and Luckow. The method is carried out in 60mm tissue-culture dishes which are seeded with $2x10^6$ cells. Serial dilutions of the virus stock are added to the attached cells and the mixture incubated with rocking to allow the virus to adsorb to individual cells. An agar overlay is added and plates incubated for 5 - 7 days at 27C. Staining of viable cells with neutral red revealed circular plaques resulting which were counted to give the virus titer.

Coinfection of Cells for Protein Production

Uninfected SF21 Cells were grown in a 60L ABEC bioreactor containing 40L of SF900 II medium. Temperature was controlled at 27C and the dissolved oxygen level was maintained at 50% of saturation by controlling the flowrate of oxygen in the inlet gas stream. When a density of $2x10^6$ cells/mL was reached, the cells were concentrated within the bioreactor to a volume of 20L using a low shear steam sterilizable pump and a with tangential flow filtration device with Millipore Prostak 0.65 micron membranes. After concentration fresh sterile growth medium is slowly added to the bioreactor while the filtration system continues to remove the spent growth medium by diafiltration. After two volume exchanges (40L) have been carried out an additional 20L of fresh medium was added to the bioreactor to resuspend the cells to the original volume of 40L. The cell density was determined once again by counting viable cells using a hemacytometer.

All the soluble receptor-Ig chimeric genes may be engineered in plasmid vectors including, but not limited to, vectors suitable for mammalian expression (Cos monkey kidney cells, Chinese Hamster Ovary cells [CHO], and ras-transformed fibroblasts [MG-ras]) and include a Kozak sequence (CGC CGC CAC CAT GGT G) [SEQ ID NO: 3] at the beginning of each chimeric gene for efficient translation. Engineering was performed using standard genetic engineering methodology. Each construct was verified by DNA sequencing, mammalian expression followed by western blotting with suitable antibodies, biophysical assays that determine ligand binding and dissociation, and by growth inhibition assays (XG-1, as described later). Since the domains utilized to engineer these chimeric proteins are flanked by appropriate restriction sites, it is possible to use these domains to engineer other chimeric proteins, including chimeras employing the extracellular domains of the receptors for factors such as IL-1, IL-2, IL-3, IL-4, IL-5, GM-CSF, LIF, IL-11, IL-15, IFNγ, TGFβ, and others. The amino acid coordinates for each component utilized in making the IL-6 traps are listed below (Note: numbering starts with the initiating methionine as #1; long sequences are listed using the single letter code for the twenty amino acids):

(a) Constructs employing human gp130:

(i) **gp130-Cγ1** was engineered by fusing in frame the extracellular domain of gp130 (amino acids 1 to 619) to a Ser-Gly bridge, followed by the 330 amino acids which comprise Cγ1 and a termination codon (Figures 9A and 9B [SEQ ID NO: 9]).

(ii) **gp130-J-Cγ1** was engineered in the same manner as gp130-Cγ1 except that a J-peptide (amino acid sequence: GQGTLVTVSS) [SEQ ID NO. 4] was inserted between the Ser-Gly bridge and the sequence of Cγ1 (see Figures 9A and 9B [SEQ ID NO: 9]).

(iii) **gp130Δ3fibro-Cγ1** was engineered by fusing in frame the extracellular domain of gp130 without its three fibronectin-like domains (Figure 10) [SEQ ID NO: 10]. The

remaining part of this chimeric protein is identical to gp130-Cγ1.

(iv) **gp130-J-CH1** was engineered in a manner identical for that described for gp130-Cγ1, except that in place of the Cγ1 region only the CH1 part of Cγ1 has been used (Figure 11). [SEQ ID NO: 11] The C-terminal domain of this construct includes the part of the hinge that contains the cysteine residue responsible for heterodimerization of the heavy chain of IgG with a light chain. The part of the hinge that contains the two cysteines involved in Cγ1 homodimerization has been deleted along with the CH2 and CH3 domains.

(v) **gp130-Cγ4** was engineered in a manner identical to that described for gp130-Cγ1, except that Cγ4 was used in place of Cγ1 (Figure 12). [SEQ ID NO: 12] In addition, an *Rsr*II DNA restriction site was engineered at the hinge region of the Cγ4 domain by introducing two silent base mutations. The *Rsrs*II site allows for other desired genetic engineering manipulations, such as the construction of the CH1 equivalent of gp130-Cγ4.

(vi) **gp130-κ** was engineered in a manner identical to that described for gp130-Cγ1, except that the constant region of the κ light chain of human Ig was used in place of Cγ1 (Figure 13). [SEQ ID NO: 13]

(vi) **gp130-J-κ** was engineered in a manner identical to that described for gp130-J-κ, except that a j-peptide (amino acid sequence: TFGQGTKVEIK) [SEQ ID NO: 5] was inserted between the Ser-Gly bridge and the κ-region.

(viii) **gp130-λ** was engineered in a manner identical to that described for gp130-Cγ1, except that the constant region of the λ light chain (Cheung, et al., Journal of Virology 66:6714-6720 (1992) of human Ig was used in place of Cγ1 (Figure 14). [SEQ ID NO: 14]

Constructs employing human IL-6Ra:

(i) **IL6R-Cγ1** was engineered by fusing in frame amino acids 1 to 358 of IL-6Rα (Yamasaki et al., Science 241:825-828 (1988), which comprise the extracellular

domain of IL-6Rα (Figure 15 [SEQ ID NO: 15]), to an Ala-Gly bridge, followed by the 330 amino acids which comprise Cγ1 and a termination codon.

(ii) **IL6R-κ** was engineered as described for IL6R-Cγ1, except that the κ-domain (Figure 13 [SEQ ID NO: 13]) utilized for gp130-κ was used in place of Cγ1.

(iii) **IL6R-j-κ** was engineered as described for IL6R-κ except that the j-peptide described for gp130-j-κ was placed between the Ala-Gly bridge and the κ-domain.

(iv) Three additional constructs, **IL6R313-Cγ1**, **IL6R313-κ**, and **IL6R313-j-κ**, were engineered as using a truncated form of IL-6Ra comprised of amino acids 1 to 313 (Figure 16 [SEQ ID NO: 16]). Each of these constructs were made by fusing in frame IL6R313 with a Thr-Gly bridge followed by the Cγ1, κ-, and j-κ-domains described above. These constructs were engineered in order to complement the gp130Δ3fibro-derived constructs.

Expression and purification of ligand traps

To produce covalently linked heterodimers of soluble gp130 and soluble IL-6Rα, gp130-Ig chimeric proteins were co-expressed with appropriate IL-6Rα-Ig chimeric proteins in complementing pairs. Co-expression was achieved by co-transfecting the corresponding expression vectors into suitable mammalian cell lines, either stably or transiently. The resulting disulfide-linked heterodimers were purified from conditioned media by several different methods, including but not limited to affinity chromatography on immobilized Protein A or Protein G, ligand-based affinity chromatography, ion exchange, and gel filtration.

An example of the type of methods used for purification of a heavy/light receptor fusion protein is as follows: gp130-Cγ1•IL-6Rα-κ was expressed in COS cells by co-transfecting two different vectors, encoding gp130-Cγ1 and IL-6Rα-κ respectively. Serum-free conditioned media (400 ml) were collected two days post-transfection and Cγ1-

bearing proteins were purified by affinity chromatography over a 1ml Protein A Sepharose (Pharmacia). The material generated in this step was further purified by a second affinity chromatography step over a 1 ml NHS-activated Sepharose (Pharmacia) which was derivatized with recombinant human IL-6, in order to remove gp130-Cγ1 dimer from gp130-Cγ1•IL-6Rα-κ complexes (the gp130-Cγ1 dimer does not bind IL-6) . Proteins generated by this method were more than 90% pure, as evidenced by SDS-PAGE followed by silver-staining (Figure 17). Similar protocols have been employed successfully towards the purification of other heavy/light receptor heterodimers.

RESULTS

Biological activity of immunoglobulin heavy/light chain receptor fusion antagonists

The purified ligand traps were tested for their ability to bind IL-6 in a variety of different assays. For example, the dissociation rate of IL-6 bound to the ligand trap was measured in parallel with the dissociation rate of IL-6 from the anti-IL-6 monoclonal neutralizing antibody B-E8 [Brochier, et al., Int. J. Immunopharmacology 17:41-48 (1995), and references within]. An example of this type of experiment is shown In Figure 18. In this experiment 20 pM ^{125}I-IL-6 (1000 Ci/mmol; Amersham) was preincubated with 500 pM of either gp130-Cγ1•IL-6Rα-κ or mAb B-E8 for 20 hours. At this point a 1000-fold excess (20 nM) of "cold" IL-6 was added. Periodically, aliquots of the reaction were removed, the ligand trap or B-E8 were precipitated with Protein G-Sepharose, and the number of cpm of ^{125}I-IL-6 that remained bound was determined. Clearly, the dissociation rate of human ^{125}I-IL6 from the ligand trap was very slow - after three days, approximately 75% of the initial counts were still bound to the ligand trap. In contrast, less than 5% of the counts remained associated with the antibody after three days. This result demonstrates that the dissociation rate of the ligand from these ligand traps is very slow.

In a different set of experiments the ability of the ligand traps to

multimerize in the presence of ligand was tested. An example of this is shown on Figures 19A and 19B. IL-6-induced association of gp130-Fc•IL-6Rα-Fc with gp130-C_H1•IL-6Rα-κ was determined by testing whether gp130-C_H1•IL-6Rα-κ, which does not by itself bind protein A, could be precipitated by protein A-Sepharose in the presence of gp130-Fc•IL-6Rα-Fc in an IL-6-depended manner (Figures 9A and 9B [SEQ ID NO: 97]). Precipitation of gp130-C_H1•IL-6Rα-κ by Protein A-Sepharose was determined by western blotting with an anti-kappa specific HRP conjugate, which does not detect gp130-Fc•IL-6Rα-Fc. gp130-C_H1•IL-6Rα-κ could be precipitated by Protein A-Sepharose only when both gp130-Fc•IL-6Rα-Fc and IL-6 were present. This result conclusively indicates that IL-6 can induce ligand trap multimerization, and further indicate that the ligand trap can mimic the hexameric cytokine•Rα•signal transducer complex (Figure 1). Ligand-induced multimerization may play a significant role in the clearance of cytokine•ligand trap complexes *in vivo*.

The biological activity of the different ligand traps may be further tested in assays which measure ligand-depended cell proliferation. Several cell proliferation assays exist for IL-6 and they employ cell lines such as B9, CESS, or XG-1. An example of this type of assay using the XG-1 cell line is presented below: XG-1 is a cell line derived from a human multiple myeloma (Zhang, et al., Blood 83:3654-3663 (1994). XG-1 depends on exogenously supplied human IL-6 for survival and proliferation. The EC_{50} of IL-6 for the XG-1 line is approximately 50 pmoles/ml. The ability of several different IL-6 traps to block IL-6-depended proliferation of XG-1 cells was tested by incubating increasing amounts of purified ligand traps with 50 pg/ml IL-6 in XG-1 cultures. The ligand traps which were tested had been expressed and purified by methods similar to those described above. All of the ligand traps tested were found to inhibit IL-6-dependent proliferation of XG-1 in a dose

(corresponding to the amino acids 1-358) from the Genbank sequence, AB006357, were cloned. For the IL-1RI, nucleotides 55 through 999 (corresponding to the amino acids 19-333) from the Genbank sequence, X16896, were cloned.

EXAMPLE 6 - PRODUCTION OF FUSION POLYPEPTIDES (CYTOKINE TRAPS)

The nucleotide sequences encoding the cytokine traps were constructed from the individual cloned DNAs (described *supra*) by standard cloning and PCR techniques. In each case, the sequences were constructed in frame such that the sequence encoding the first fusion polypeptide component was fused to the sequence encoding the second fusion polypeptide component followed by an Fc domain (hinge, CH2 and CH3 region of human IgG1) as the multimerizing component. In some cases extra nucleotides were inserted in frame between sequences encoding the first and second fusion polypeptide components to add a linker region between the two components (See Figs. 21A-21D - trap 424; Figs. 24A-24F - trap 412; and Figs. 26A-26E - trap 569).

For the IL-4 traps, 424 (Figs. 21A-21D), 603 (Figs. 22A-22D) and 622 (Figs. 23A-23D), the IL-2Rγ component is 5′, followed by the IL4Rα component and then the Fc component. For the IL-6 traps, 412 (Figs. 24A-24F) and 616 (Figs. 25A-25F), the IL-6Rα component is 5′ followed by the gp130 component and then the Fc domain. For the IL-1 trap 569 (Figs. 26A-26E), the IL-1RAcP component is 5′ followed by the IL-1RI component and then the Fc domain. The final constructs were cloned into the mammalian expression vector pCDNA3.1 (STRATAGENE).

In the 569 sequence (Figs. 26A-26E) [SEQ ID NO: 27], nucleotides 1-1074 encode the IL1RAcP component, nucleotides 1075 -1098 encode a linker region, nucleotides 1099-2043 encode the IL1RI component and nucleotides 2044-2730 encode the Fc domain.

In the 412 sequence (Figs. 24A-24F) [SEQ ID NO: 23], nucleotides 1-993 encode the IL6Rα component, nucleotides 994-1023 encode a linker region, nucleotides 1024-2814 encode the gp130 component and nucleotides 2815-3504 encode the Fc domain.

In the 616 sequence (Figs. 25A-25F) [SEQ ID NO: 25], nucleotides 1-993 encode the IL6Rα component, nucleotides 994-2784 encode the gp130 component and nucleotides 2785-3474 encode the Fc domain.

In the 424 (Figs. 21A-21D) [SEQ ID NO: 17] and 622 (Figs. 23A-23D) [SEQ ID NO: 21] sequences, nucleotides 1-762 encode the IL2Rγ component, nucleotides 763-771 encode a linker region, nucleotides 772-1395 encode the IL4Rα component and nucleotides 1396-2082 encode the Fc domain.

Finally, in the 603 sequence (Figs. 22A-22D) [SEQ ID NO: 19], nucleotides 1-762 encode the IL2Rγ component, nucleotides 763-1386 encode the IL4Rα component and nucleotides 1387-2073 encode the Fc domain.

DNA constructs were either transiently transfected into COS cells or stably transfected into CHO cells by standard techniques well known to one of skill in the art. Supernatants were collected and purified by protein A affinity chromatography and size exclusion chromatography by standard techniques. (See for example Harlow and Lane, Antibodies - A Laboratory Manual, Cold Spring Harbor Laboratory, 1988).

6. Dispense 50µl of the cell suspension (40000 cells) into all wells of the plates. Total volume should now be 100µl/well.

7. Incubate the plate at 37°C for 68 hours in a humidified 5% CO_2 incubator.

C. Color Development

8. At 68 hours add 15µl of the dye solution to each well.

9. Incubate the plate at 37°C for 4 hours in a humidified 5% CO_2 incubator.

10. After 4 hours, add 100µl of the solubilization solution to each well. Allow the plate to stand overnight in a sealed container to completely solubilize the formazan crystals.

11. Record the absorbance at 570/650nm.

Results:

Figure 29 shows that the IL6 trap (6SC412 IL6R-scb-gpx-FcΔC1) described in Figs. 24A-24F (SEQ ID NOS: 23 and 24) is a better antagonist of IL-6 in the XG1 bioassay than the neutralizing monoclonal antibody to human IL-6 - BE8.

Example 9: MRC5 Bioassay for IL1 Traps

MRC5 human lung fibroblast cells respond to IL-1 by secreting IL-6 and thus were utilized to assay the ability of IL-1 traps to block the IL-1-dependent production of IL-6. IL1 Trap 1SC569 (Figs. 26A-26E) (SEQ ID NOS: 27 and 28) was tested against IL-1-RI.Fc which is the extracellular domain of the IL-1 Type I receptor fused to an Fc domain.

MRC5 cells are suspended at 1 x 10^5 cells per ml in medium and 0.1 ml of cells are plated (10,000 cells per well) into the wells of a 96 well tissue culture plate. Plates are incubated for 24 hours at 37°C in a humidified 5% CO_2 incubator.

IL-1 trap and recombinant human IL-1 at varying doses are pre-incubated in a 96 well tissue culture dish and incubated for 2 hours at 37°C. 0.1 ml of this mixture is then added to the 96 well plate containing the MRC5 cells such that the final concentration of IL-1 Trap is 10nM and the final concentrations of the IL-1 ranges from 2.4 pM to 5nM. Control wells contain trap alone or nothing.

Plates are then incubated at 37°C for 24 hours in a humidified 5% CO_2 incubator. Supernatant is collected and assayed for levels of IL-6 using R&D Systems Quantikine Immunoassay Kit according to the manufacturer's instructions.

Results: [SEQ ID NOS: 27 and 28]

Figure 30 shows that the trap 569 (Figs. 26A-26E) is able to antagonize the effects of IL-1 and block the IL-6 production from MRC 5 cells upon treatment with IL-1. At a concentration of 10nM, the trap 569 is able to block the production of IL-6 up to an IL-1 concentration of 3nM. In contrast, the IL-1RI.Fc is a much poorer antagonist of IL-1. It is only able to block the effects of IL-1 up to about 10-20 pM. Thus, the trap 569 is approximately 100x better at blocking IL-1 than IL1RI.Fc.

amino acid sequence of the IL-4Rα.IL-13Rα1.Fc single chain trap is set forth in Figure 31.

b. IL-13Rα1.IL-4Rα.Fc

The IL-13Rα1 extracellular domain (including the signal peptide, therefore nts #34-1062; amino acid #1-343) was amplified by standard PCR techniques and ligated into an expression vector, pJFE14, to create an in-frame fusion upstream of the human IL-4Ra (nucleotides 236 through 868 (corresponding to the amino acids 21-231) from the Genbank sequence X52425) and human Fc. A ten amino acid linker with the amino acid sequence GAPSGGGGRP (SEQ ID NO:6) was constructed in frame between the IL-4Rα and the IL-13Rα1. The human IL-13Rα1 sequence was verified. The construct encoding this single chain trap was transfected into COS cells and protein expression was verified by Western Blot analysis of the cell media. The nucleotide and amino acid sequence of the IL-13Rα1.IL-4Rα.Fc single chain trap is set forth in Figures 32A-32G (SEQ ID NOS: 31 and 32).

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.